P-Cadherin Overexpression Is an Indicator of Clinical Outcome in Invasive Breast Carcinomas and Is Associated with *CDH3* Promoter Hypomethylation

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Abstract

Purpose: P-cadherin overexpression has been reported in breast carcinomas, where it was associated with proliferative high-grade histological tumors. This study aimed to analyze P-cadherin expression in invasive breast cancer and to correlate it with tumor markers, pathologic features, and patient survival. Another purpose was to evaluate the P-cadherin promoter methylation pattern as the molecular mechanism underlying this gene regulation.

Experimental Design: Using a series of invasive breast carcinomas, P-cadherin expression was evaluated and correlated with histologic grade, estrogen receptor, MIB-1, and p53 and c-erbB-2 expression. In order to assess whether P-cadherin expression was associated with changes in *CDH3* promoter methylation, we studied the methylation status of a gene 5′-flanking region in these same carcinomas. This analysis was also done for normal tissue and for a breast cancer cell line treated with a demethylating agent.

Results: P-cadherin expression showed a strong correlation with high histologic grade, increased proliferation, c-erbB-2 and p53 expression, lack of estrogen receptor, and poor patient survival. This overexpression can be regulated by gene promoter methylation because the 5-Aza-2′-deoxycytidine treatment of MCF-7/AZ cells increased P-cadherin mRNA and protein levels. Additionally, we found that 71% of P-cadherin-negative cases showed promoter methylation, whereas 65% of positive ones were unmethylated ($P = 0.005$). The normal P-cadherin-negative breast epithelial cells showed consistent *CDH3* promoter methylation.

Conclusions: P-cadherin expression was strongly associated with tumor aggressiveness, being a good indicator of clinical outcome. Moreover, the aberrant expression of P-cadherin in breast cancer might be regulated by gene promoter hypomethylation.

Cadherins are cell-cell adhesion glycoproteins that form calcium-dependent intercellular junctions and play an essential role in morphogenesis and in the development and maintenance of adult tissues and organs (1). During embryogenesis, the cell expression of specific cadherins results in homophilic interactions that are critical in the process of cell sorting and tissue stratification (2–4). Alterations in these cellular attachments play an important role in cell destabilization and may modify the carefully regulated differentiation process of the epithelial structures (5, 6). For this reason, the functional loss or overexpression of cadherins and the molecular mechanisms underlying the control of the genes codifying these proteins have been implicated in carcinogenesis (7).

The cadherin family is subdivided into various subfamilies, including the classical E-, P-, and N-cadherins, each demonstrating a specific tissue distribution (8). Although E-cadherin is expressed in all epithelial tissues, the expression of P-cadherin is only restricted to the basal or lower layers of stratified epithelia, including prostate and skin, and also to the breast myoepithelial cells (6, 9). This unique distribution of P-cadherin suggests that, in addition to maintaining cellular adhesion, this molecule may also have other unknown functions, which can be important in cell differentiation and proliferation (5, 6).

Up-regulation of P-cadherin has been shown in several lesions, including breast cancer, in which there is usually down-regulation of E-cadherin (10). Breast carcinomas show aberrant P-cadherin expression in ~30% of the cases and has been reported as a prognostic marker of poor outcome in patients (11, 12). The differential pattern of P-cadherin expression in

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breast cancer development, coupled with its possible prognostic value, prompted us to investigate its expression in a series of invasive breast carcinomas.

Furthermore, we have previously found that P-cadherin aberrant expression results from a lack of estrogen receptor-α (ER-α) signaling and induces *in vitro* cell invasion in a juxtamembrane domain-dependent manner (13). Additionally, based on the fact that the 5′-flanking region (Genbank no. X95824) of the P-cadherin gene (*CDH3*) has been characterized as a CpG island (14), we thought that promoter methylation could be a putative molecular mechanism responsible for its transcriptional regulation.

This hypothesis is also supported by the fact that the same mechanism of regulation was shown for the E-cadherin gene. Aberrant methylation across the promoter region of this gene results in a selective inactivation of its transcription (15, 16). E- and P-cadherin genes exhibit a similar genomic organization, both containing 16 exons and a similar promoter region (17). The localization of the human P-cadherin gene is at 32 kb upstream of the human E-cadherin gene, also mapping to chromosome 16q22.1, showing the evolutionary conservation of the tandem arrangement of two genes encoding cell adhesion molecules, suggesting that the close proximity of these genes may be important for their regulation (18).

The 5′-flanking region of the E-cadherin gene has been sequenced and found to be extremely CG-enriched, meeting the criteria for a "CpG island" (19, 20). Methylation of E-cadherin has been shown in breast cancer and in some other cancers and cell lines (16, 21–24), although this functional block of E-cadherin expression can be removed with exposure of cell lines to demethylating agents *in vitro* (25–27). Additional factors regarding the E-cadherin gene, which seem important to transcriptional regulation, include a palindromic sequence E-pal (where transcriptional repressors as Snail and Slug bind) and a Sp-1 binding site (19).

In contrast, the comparison of the 5′-flanking sequence of the human P-cadherin gene with the one for E-cadherin shows no homology for this palindromic sequence E-pal (17). In the P-cadherin promoter, there is conservation of a CAAT box, with no TATA box, and three E boxes (helix-loop-helix binding motif). A putative Sp-1 binding site is also conserved (14). An *Alu* repeat is present ~700 bp upstream from the translation start site, and analogous to the human E-cadherin sequence, shows a CG-rich region characteristic of a CpG island (14).

Genes that show a CpG island within their promoter region are normally regulated by methylation. Few studies describing P-cadherin promoter methylation status have been published thus far and, to the best of our knowledge, none have been done in human breast cancer specimens. However, cytosine methylation of this region was described in P-cadherin-nonexpressing prostate cancer cell lines (LNCaP, TSU-PR1, and DuPRO) but not in cell lines expressing this gene (PC3, DU145, and PPC1; refs. 14, 19). Simultaneously, a recent study describing epigenetic silencing of E- and P-cadherin gene expression in human melanoma cell lines was reported, in which methylation-specific PCR analysis revealed that P-cadherin seems to be silenced by methylation events (28).

Although these studies point to cytosine methylation as a possible mechanism of P-cadherin expression regulation, Jarrard et al. claim that other mechanisms should be able to regulate the consistent loss of P-cadherin expression in *in vivo* prostate cancer specimens. In contrast with breast cancer models, these authors found that P-cadherin is restricted to basal epithelial cells in normal prostate samples, but is down-regulated in prostatic intraepithelial neoplasia and is absent in all prostate cancer specimens analyzed. Additionally, the lack of expression was not associated with methylation of the P-cadherin promoter (14).

Based on all these results, we decided to evaluate if the promoter methylation could be the molecular mechanism responsible for the transcription regulation of the *CDH3* gene, using a series of invasive breast carcinomas. These results were compared with normal breast tissue, and confirmed in a breast cancer cell line treated with a demethylating agent.

Materials and Methods

Breast tumor samples

Formalin-fixed paraffin-embedded blocks of 150 invasive breast carcinomas were retrieved from the histopathology files at the Department of Pathology, Hospital Xeral Cíes, Vigo, Spain, selected from a cohort of patients with follow-up information. From all cases, we analyzed the available relevant data, including age, tumor size, mitotic index, axillary metastasis, tumor grade, ER-α status, MIB-1, c-erbB-2, and p53 expression. The mean age of the patients at diagnosis was 56 years (range, 28-82 years old). The size of the tumors ranged from 1 to 10 cm (mean, 2.8 ± 1.5 cm). ER-α was evaluated in 150 tumors, where 100 cases were positive. MIB-1 expression was assessed in 147 cases, 70 with <15% of positive cells and 77 with >15% of positive cells. C-erbB-2 expression was analyzed in 139 cases and p53 expression in 145 cases, 34 cases were positive for c-erbB-2 and 66 were positive for p53. As a measure of prognosis, we analyzed the clinical data concerning disease-free survival, defined as the time from diagnosis to first recurrence or last contact, and the overall survival, defined as the time from diagnosis to death by breast cancer or last contact.

Immunohistochemistry

Immunohistochemical studies of representative sections were carried out using standard methods. Sections cut 2 μm thick were mounted on gelatin (Merck, Merck KgaA, Darmstadt, Germany) and chromium (III) potassium sulfate 12-hydrate–coated slides (Merck), dried overnight at 37°C, deparaffinized with xylene and hydrated. An antigen retrieval method was carried out by microwave treatment, in a 0.05% detergent solution for E- and N-cadherin antibodies (10 minutes), and with a 10 mmol/L citrate buffer, pH 6.0 (DAKO Corporation, Carpinteria, CA) for P-cadherin antibody (30 minutes). Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 10 minutes. Sections were submitted to protein blockage with a specific serum (Ultravision block, LabVision Corporation, Fremont, CA) and incubated with the monoclonal primary antibody for 30 minutes at room temperature (E-cadherin, HECD-1, 1:200, Zymed Laboratory, San Francisco, CA; P-cadherin, clone 56, 1:50, Transduction Laboratories, Lexington, KY; and N-cadherin, clone 3B9, 1:400, Zymed Laboratory). The other steps of immunohistochemical staining were done using standard protocols on an automated LabVision Autostainer (LabVision Corporation), with a specific kit based on streptavidin-biotin-peroxidase method. Subsequently, the sections were counterstained with Mayer's hematoxylin, dehydrated, cleared, and mounted.

Positive and negative controls were included with each batch of staining to ensure consistency between consecutive runs. Paraffin sections of normal skin tissue were used as positive controls for E-cadherin, normal breast tissue was used for P-cadherin, and cardiac muscle was used for N-cadherin. Immunohistochemical results were not assessed in some cases (49 for E-cadherin, 3 for P-cadherin, and 8 for N-cadherin) because there was no more tumor material available.

Quantification of immunostaining

All tumors presenting an unequivocal membranous staining for cadherin in at least 10% of the neoplastic cells were scored as positive. Cells with cytoplasmic expression alone were not considered. The assessment of immunohistochemical results was based on a semiquantitative evaluation, which did not include staining intensity, as previously reported (29).

Cell culture and treatments

The human MCF-7/AZ breast cancer cell line was obtained from Prof. Marc Mareel (Laboratory of Experimental Cancerology, Ghent University, Belgium) and routinely maintained at 37°C and 10% CO_2, in 50% DMEM/50% Ham's F12 media (Invitrogen, Merelbeke, Belgium), supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 100 IU/mL penicillin, and 100 g/mL streptomycin (Invitrogen).

These cells were incubated during 5 days with 2.5 μmol/L of 5-Aza-2′-deoxycytidine (Sigma Chemical Co., St. Louis, MO), which reduces the level of 5-methylcytosine in DNA, or just with DMSO (which was the vehicle of this drug) for use as a control. After 5 days, RNA and protein cell lysates were isolated from these cells.

Reverse transcription-PCR analysis

Reverse transcription-PCR experiments were done using total RNA, which was extracted from $\sim 5 \times 10^6$ cells using the Qiagen RNeasy kit (Qiagen, Chatsworth, CA). One microgram of total RNA was reverse-transcribed with oligo(dT) primers using the Qiagen RT kit (Qiagen) according to the manufacturer's instructions. P-cadherin cDNA was amplified using the sense primer 5′-ACGAAGACACAAGAGAGATTGG and the antisense primer 5′-CGATGATGGAGATGTTCATGG, in order to generate a 287-bp product. PCRs were done in 250 ng template cDNA using the Qiagen Taq PCR kit (Qiagen) according to the manufacturer's instructions. Reactions were done in a Minicycler (Biozym, Landgraaf, the Netherlands) with an initial denaturation at 94°C for 3 minutes; 20, 30, or 35 cycles of 94°C for 50 seconds (denaturation), 55°C for 50 seconds (annealing), and 72°C for 1 minute (elongation), followed by a final extension at 72°C for 10 minutes.

Western blot analysis

All lysates were made of cells ~90% confluence, which were washed thrice with PBS. Cells were lysed with PBS containing 1% Triton X-100, 1% Nonidet P-40 (Sigma), and the following protease inhibitors: aprotinin (10 μg/mL), leupeptin (10 μg/mL; ICN Biomedicals, Costa Mesa, CA), phenylmethylsulfonyl fluoride (1.72 mmol/L), NaF (100 μmol/L), $NaVO_3$ (500 μmol/L), and $Na_4P_2O_7$ (500 μg/mL; Sigma). After clearing the lysates, protein concentration was determined using the Rc Dc protein assay (BioRad, Richmond, CA), and samples were prepared such that equal amounts of protein were to be loaded. Sample buffer (Laemmli) with 5% 2-mercaptoethanol and 0.012% bromophenol blue was added, followed by boiling for 5 minutes and separation of proteins by gel electrophoresis on an 8% polyacrylamide gel and transfer onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Quenching and immunostaining of the blots were done in 5% nonfat dry milk in PBS containing 0.5% Tween 20. The membranes were quenched for 1 hour, incubated with primary antibody for P-cadherin (clone 56, 1:500, Transduction Laboratories) for 1 hour, washed four times for 10 minutes, incubated with horseradish peroxidase–conjugated secondary antibody for 45 minutes, and washed six times for 10 minutes. Detection was done using enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) as a substrate.

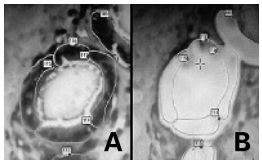

Fig. 1. Illustration of a normal mammary duct selected for laser tissue microdissection: *A,* only clearly identifiable epithelial cells layer were cautiously selected. *B,* the epithelial cell clusters were laser-cut and catapulted for a special vial for DNA extraction.

Laser-assisted tissue microdissection

To analyze the methylation pattern in normal epithelia, five frozen normal breast tissues were selected from the archive of the Department of Pathology, Hospital São João, Porto, Portugal. These frozen samples were included in ornithine carbamyl transferase solution (Bright, Huntingdon, England) and sections 10 μm thick were placed in specific slides for laser microdissection. The slides were stained with H&E and visualized in a Laser Microdissector Microscope (PALM, Bernried, Germany). Breast normal glandular epithelia were identified in the sections and only prominent epithelial cells were selected for microdissection (Fig. 1A). In all cases, >10,000 epithelial cells were cautiously laser-cut and laser pressure–catapulted for a specific vial (Fig. 1B), from which DNA extraction was done according to Qiagen protocols for DNA extraction from laser tissue microdissection.

Methylation-specific PCR analysis

In order to optimize the methylation-specific PCR technique, we used genomic DNA extracted from nine frozen breast tumor tissues retrieved from the Portuguese Oncology Institute of Coimbra (IPOFG-CROC, Portugal). The tumor tissues were macerated and genomic DNA extraction was done according to the standard protocol for human tissues—Genomic DNA from Tissue Kit (NucleoSpin Tissue, Macherey-Nagel, GmbH & Co., KG, Düren, Germany). Genomic DNA (200 ng/μL) was converted by bisulfite treatment as described below. Because we studied P-cadherin protein expression in these frozen tumor cases, these methylation-specific PCR results were also considered for statistical analysis, together with 55 cases from the series of paraffin-embedded samples, where tumor material was still available to perform DNA extraction.

DNA extraction

Fifty-five paraffin-embedded samples were cut to 10-μm-thick sections and, after identification of the tumor areas of interest, these were carefully microdissected. The samples were then placed into microcentrifuge tubes with a freshly prepared lysis buffer/proteinase K mixture, and incubated overnight at 56°C until complete lysis of the fragments. The DNA extraction was done according to the same standard protocol for human tissues previously referred to. DNA from all 64 samples (55 paraffin-embedded and 9 frozen cases) was quantified, and wild-type DNA from each case was amplified using P-cadherin-specific primers. Genomic blood DNA was used as wild-type positive control (Fig. 2A).

Methylation analysis

Bisulfite treatment. Sodium bisulfite conversion of P-cadherin 5′ CpG island was done in 3 μg of genomic DNA from the total 64 tumor cases. This same treatment was applied to DNA extracted from the five normal breast tissues that were laser-microdissected. Briefly, and based on the standard protocol described elsewhere (30), DNA was denatured by incubation with NaOH (final concentration, 0.2 mol/L), for 20 minutes at 50°C. To function as a carrier, 1 μg of salmon sperm DNA (Stratagene, La Jolla, CA) was mixed with the initial human DNA. A freshly made sodium bisulfite solution [2.5 mol/L sodium bisulfite (Sigma), 125 mmol/L hydroquinone (Sigma), and NaOH 2 mol/L (pH 5.0)] was added to the DNA sample, in order to convert the unmethylated cytosines to uracils. This conversion allows the distinction between methylated and unmethylated DNA. After 3 hours of incubation in the dark at 70°C, converted DNA was purified, using a commercial Wizard DNA purification resin, as described by the manufacturer (Promega Corporation, Madison, WI). Finally, the purified DNA was eluted into 45 μL of preheated (80°C) water.

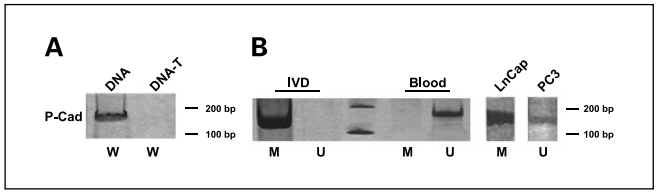

Fig. 2. Wild-type and methylation control patterns of the analyzed P-cadherin promoter region (W, wild-type; M, methylated; U, unmethylated): *A,* wild-type pattern of genomic DNA untreated and treated with bisulfite (DNA-T). As expected, P-cadherin wild-type primers do not recognize the promoter site of bisulfite-transformed DNA. *B,* on the left, the blood DNA treated with CpG methylase M.SssI (*in vitro* DNA) was only amplified with P-cadherin primers specifically designed for methylated promoter region after the bisulfite treatment. Bisulfite-treated DNA from blood was used as a positive control for the unmethylated promoter region. On the right, bisulfite-treated DNA from LnCap and PC3 cell lines were also used as P-cadherin methylation and unmethylation positive controls, respectively.

Modification was completed by NaOH treatment (final concentration, 0.3 mol/L) for 10 minutes at room temperature, followed by neutralization reaction with 75 μL of ammonium acetate 6 mol/L. DNA precipitation was done by adding 2 μL of glycogen (5 mg/mL; MBI Fermentas), and after pellet washing with 70% ethanol, DNA was dried, resuspended in 40 μL of water and stored at −20°C.

Methylation-specific PCR analysis. After the bisulfite treatment, all samples were amplified by PCR. For a final volume of 25 μL of PCR reaction mix, 400 ng of bisulfite-modified DNA was added to the PCR mix containing 1× PCR buffer [16.6 mmol/L ammonium sulfate, 67 mmol/L Tris (pH 8.8), 6.7 mmol/L $MgCl_2$, and 10 mmol/L 2-mercaptoethanol], deoxynucleotide triphosphates (each at 1 mmol/L), 0.6 μmol/L primers, 2% DMSO and 1 unit Platinum Taq DNA polymerase (Life Technologies, Inc., Rockville, MD). The gene-specific primer sequences for both methylated and unmethylated P-cadherin promoter were, respectively: P-cad-M (sense 5′-GGCGGGATTTCGTGGCGT; antisense 5′-ATAAAACAACTACCGCGACG); P-cad-U (sense 5′-GGTGGGATTTTGTGGTGTTG; antisense 5′-ATAAAACAACTACCACAACAACA). The annealing temperature was 55°C, generating a 140-bp product. Reactions were hot-started at 95°C for 5 minutes and PCR amplification was carried out in a thermocycler for 38 cycles (30 seconds at 95°C, 30 seconds at 55°C, the annealing temperature, and 45 seconds at 72°C). A final extension was done at 72°C for 10 minutes. Negative controls, without DNA, were done for all sets of PCRs. Each PCR product was directly loaded into a nondenaturing 6% polyacrylamide gel, and stained with silver nitrate. As a positive methylation control, we used blood DNA treated with CpG methylase M.SssI (BioLabs Inc., New England; *in vitro* DNA), and blood bisulfite-treated DNA was used as a positive unmethylated control (Fig. 2B). Genomic DNA was also obtained from human prostate cancer cell lines, for use as additional positive methylation controls: LNCaP, which present methylation within the *CDH3* promoter, and PC3, which express detectable P-cadherin and are unmethylated at *CDH3* promoter (ref. 14; Fig. 2B).

As already mentioned, we also did PCR amplification for P-cadherin promoter wild-type sequence, and the primers used were: P-cad-W (sense 5′-GGGGCGGGACCTCGTGGCGC; antisense 5′-GTGAAGCAG-CTGCCGCGACG). The annealing temperature was 67°C, generating a 142-bp product. Human DNA extracted from blood leukocytes was used as positive controls. For a final volume of 30 μL of PCR reaction mix, 200 ng of DNA was added to 1× PCR buffer [500 mmol/L KCl, 100 mmol/L Tris (pH 8.8), and 0.8% NP40], deoxynucleotide triphosphates (each at 0.2 mmol/L), 0.3 μmol/L primers, and 1 unit of Taq DNA polymerase (Amersham Pharmacia Biotech). The PCR amplification was carried out using the following conditions: 1 cycle at 95°C for 5 minutes, and 35 cycles at 95°C for 30 seconds, followed by 67°C (specific annealing temperature) for 30 seconds and 72°C for 45 seconds. Additionally, a final extension at 72°C for 10 minutes was done. Negative controls without DNA were used for all sets of PCRs. As expected, P-cadherin wild-type primers did not recognize the promoter site of bisulfite-transformed DNA (Fig. 2A). All the amplified products were directly loaded and stained as described above.

In order to classify the methylation status of the cases that were studied, the ones showing only methylated alleles were considered *methylated* (M; or with complete methylation), and the ones with only unmethylated alleles were considered *unmethylated* (U; or with complete demethylation). The cases that showed partial methylation, which means the presence of both methylated and unmethylated alleles, were also considered methylated (M), based on previous reports describing methylation-specific PCR analysis (24, 31, 32).

Statistical analysis

For statistical analysis, contingency tables and χ^2 test were done to estimate the relationship between staining patterns of the different cadherins and several of the factors analyzed, such as tumor grade, lymph node metastasis, vascular invasion, ER-α, MIB-1, c-erbB-2 and

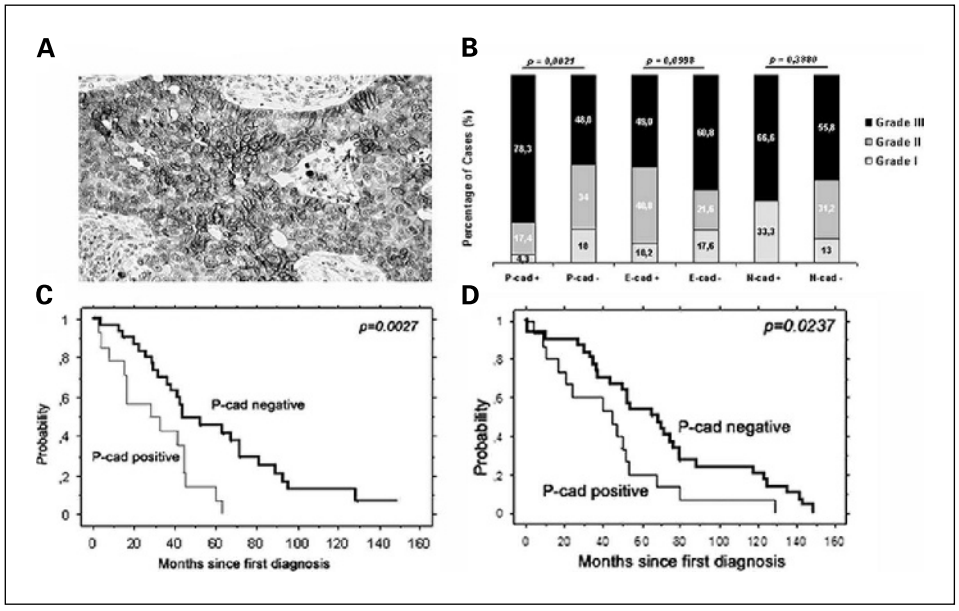

Fig. 3. *A,* strong membranous and cytoplasmic expression of P-cadherin in a high-grade invasive breast carcinoma. H&E, ×400. *B,* correlation between P-, E-, and N-cadherin expression and tumor histologic grade. *C* and *D,* Kaplan-Meier curves showing probability of disease-free survival and overall-survival, respectively, for patients with P-cadherin-positive tumors versus patients with P-cadherin-negative tumors (log-rank test).

Table 1. Correlation between P-, E-, and N-cadherin expression and ER-α, MIB-1, c-erbB-2, and p53-positive expression

	P-cad +	P-cad −	*P*	E-cad +	E-cad −	*P*	N-cad +	N-cad −	*P*
ER-α	34.8% (16 of 46)	80.2% (81 of 101)	<0.0001	75.5% (37 of 49)	61.53% (32 of 52)	0.1315	100% (3 of 3)	68.3% (95 of 139)	0.2408
MIB-1	76% (35 of 46)	42.9% (42 of 98)	0.0002	46.8% (22 of 47)	54.9% (28 of 51)	0.4233	33.3% (1 of 3)	52.2% (71 of 136)	0.5176
c-erbB-2	41.5% (17 of 41)	17.9% (17 of 95)	0.0036	32.56% (14 of 43)	20% (10 of 50)	0.1676	33.3% (1 of 3)	23.4% (30 of 128)	0.6902
p53	62.2% (28 of 45)	37.8% (37 of 98)	0.0064	39.6% (19 of 48)	50% (25 of 50)	0.3000	50% (1 of 2)	46.6% (63 of 135)	0.9253
Total	46	101		49	52		3	139	

p53 expression, and *CDH3* methylation status. ANOVA was used to investigate differences in tumor size and mitotic index. Two values were considered significantly different when $P < 0.05$. Univariate survival curves were estimated using the method of Kaplan-Meier and compared using the log-rank test. Statistical analyses were carried out using StatView 5.0 Software (SAS Institute Inc., Cary, NC).

Results

Cadherin expression and correlation with biological markers

From the 150 cases of the studied series, 149 were graded in accordance with the modified criteria of Bloom and Richardson (14, 33): 21 were classified as grade I (14.1%), 44 as grade II (29.5%), and 84 as grade III (56.4%) tumors.

P-cadherin immunoreactivity was absent in 101 (68.7%) primary breast cancers analyzed and aberrantly expressed in 46 cases (31.3%). Distribution of P-cadherin in tumor cells showed membranous staining frequently associated with cytoplasmic expression (Fig. 3A). P-cadherin expression was always found in myoepithelial cells from normal ducts/acini and from ducts containing *in situ* carcinoma, but not in acinar or ductal normal epithelial cells.

P-cadherin expression showed a statistically significant correlation with histologic grade, because this protein was essentially present in high-grade tumors (36 of 46; 78.3%), whereas grade I cases were almost all negative ($P = 0.0021$; Fig. 3B).

Statistical analysis revealed an inverse correlation between P-cadherin expression and ER-α status: 80.2% of P-cadherin-negative cases were ER-α-positive. The χ^2 test showed that this difference was statistically significant ($P < 0.0001$; Table 1). The correlation between P-cadherin and MIB-1, and c-erbB-2 and p53 expressions, was also evaluated: the majority of P-cadherin-positive cases were highly proliferative ($P = 0.0002$), and significantly related to c-erbB-2 ($P = 0.0036$) and p53 expression ($P = 0.0064$; Table 1). Additionally, an important association was observed with high mitotic index ($P = 0.0003$, data not shown). No significant correlation was found between P-cadherin expression and tumor size, axillary lymph node metastasis, and angiogenesis. Although invasive ductal carcinomas and medullary carcinomas were frequently P-cadherin-positive, a statistically significant difference with the tumor histologic type was not reached.

Concerning E-cadherin, half of the studied cases were negative (52 of 101; 51.5%), showing a clear decrease of membranous staining in the neoplastic epithelial cells. N-cadherin was only found in 2.11% (3 of 142) of the tumors, and its expression was restricted to a small population of cells with a faint membranous staining, and with cytoplasmic expression. No correlation was found between the expression of these two cadherins and any of other tumor parameters studied here (Table 1; Fig. 3B), and there were also no significant associations between the expression of cadherins.

Cadherin expression and survival analysis

The mean follow-up time was 113 ± 53 months (range, 1-176 months). There were 12 local recurrences after diagnosis,

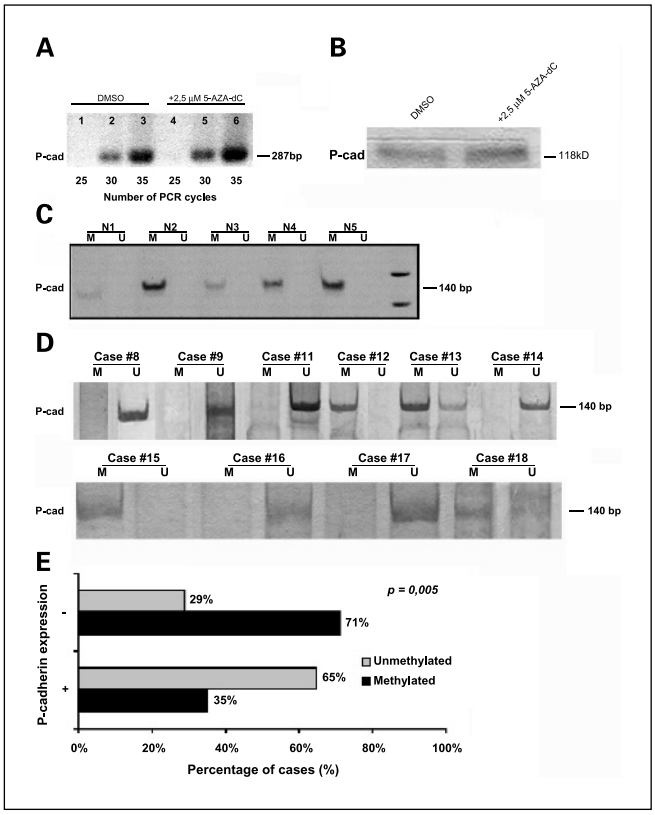

Fig. 4. *A,* reverse transcription-PCR analysis of P-cadherin mRNA levels after 5-Aza-2′-deoxycytidine treatment of MCF-7/AZ cells for 5 days. The analysis was done after the indicated number of cycles of PCR amplification. In the presence of the demethylating agent, the increased P-cad mRNA was more evident at the 30-cycle point, during the exponential phase of the PCR reaction. *B,* immunoblotting, for P-cadherin analysis, of cell lysates from MCF-7/AZ cells that had been treated with 5-Aza-2′-deoxycytidine. In comparison with the control cells, there was an increased P-cadherin expression in the MCF-7/Az cells that were incubated with the demethylating agent. *C,* methylation-specific PCR results from the five normal breast tissues, where epithelial cell layers were laser microdissected from the ducts. All the cases showed complete *CDH3* promoter methylation. *D,* examples of complete or partial methylation and unmethylation patterns: complete methylation pattern (cases 12 and 15), partial methylation pattern (cases 13 and 18), and complete unmethylation pattern (cases 8, 9, 11, 14, 16, and 17). *E,* correlation between P-cadherin expression and the methylation status of the analyzed gene promoter region. The majority of P-cadherin-negative cases were unmethylated on this promoter region ($P = 0.005$).

Table 2. *CDH3* promoter methylation status and P-cadherin expression in the 64 breast tumor samples studied by methylation-specific PCR analysis

Patient no.	*CDH3* promoter methylation		P-cadherin expression	Patient no.	*CDH3* promoter methylation		P-cadherin expression
	Alleles	Status			Alleles	Status	
#1	U/U	U	positive	#72	U/U	U	negative
#2	U/U	U	negative	#73	M/M	M	positive
#3	U/U	U	positive	#74	M/M	M	negative
#4	U/U	U	negative	#75	M/M	M	negative
#5	U/U	U	negative	#76	M/M	M	positive
#6	M/U	M	negative	#77	M/M	M	negative
#7	M/U	M	negative	#78	M/M	M	negative
#8	U/U	U	positive	#79	M/M	M	negative
#9	U/U	U	positive	#80	M/M	M	negative
#11	U/U	U	positive	#81	M/M	M	positive
#12	M/M	M	negative	#85	M/M	M	negative
#13	M/U	M	negative	#86	M/M	M	negative
#14	U/U	U	positive	#111	M/M	M	negative
#15	M/M	M	negative	#113	M/M	M	negative
#16	U/U	U	positive	#119	M/M	M	negative
#17	U/U	U	positive	#125	M/M	M	positive
#18	M/U	M	negative	#126	M/M	M	negative
#19	M/U	M	negative	#127	M/M	M	positive
#20	U/U	U	positive	#132	M/M	M	negative
#21	M/U	M	positive	#133	M/M	M	positive
#22	U/U	U	positive	#134	M/M	M	negative
#24	M/U	M	negative	#135	M/M	M	negative
#25	U/U	U	positive	#138	M/M	M	negative
#41	M/M	M	negative	#148	M/M	M	negative
#42	M/M	M	positive	#151	U/U	U	positive
#51	U/U	U	negative	#153	U/U	U	negative
#59	U/U	U	negative	#154	U/U	U	positive
#60	U/U	U	negative	#155	U/U	U	negative
#61	U/U	U	negative	#157	M/M	M	negative
#65	M/M	M	negative	#159	U/U	U	negative
#66	U/U	U	positive	#160	U/U	U	negative
#67	M/M	M	negative	#161	U/U	U	positive

45 cases with distant metastasis, and 45 deaths because of breast cancer in the cohort. Disease-free survival and overall survival differed significantly between classes of P-cadherin expression, as revealed in Kaplan-Meier plots. The probabilities of disease-free and overall survival were significantly lower for patients with P-cadherin-positive tumors ($P = 0.0027$ and $P = 0.0237$, respectively; Fig. 3C and D). The interval between diagnosis and local recurrence also showed a slight correlation with P-cadherin expression: a mean of 35 ± 20.15 months for positive and 73.6 ± 32 months for negative tumors ($P = 0.0545$). No correlation was found between E- or N-cadherin expression and disease-free and overall survival.

P-cadherin promoter methylation

MCF-7/AZ cells treated with 5-Aza-2′-deoxycytidine. To evaluate whether it would be reasonable to test our hypothesis in a breast cancer series, we treated MCF-7/AZ cells, which present lower levels of P-cadherin, with the demethylating agent 5-Aza-2′-deoxycytidine (2.5 μmol/L). After 5 days, we harvested the cells, and we analyzed P-cadherin mRNA and protein levels, by reverse transcription-PCR and Western blot, respectively. We observed an increase of both levels, suggesting that hypomethylation of the promoter accompanies transcriptional activation of the *CDH3* gene in this breast cancer cell line (Fig. 4A and B).

Normal breast epithelial cells. P-cadherin promoter methylation was analyzed in five cases of normal breast tissue, from which only epithelial cells were microdissected. Methylation of P-cadherin gene was found in all the cases analyzed, because none of the samples showed the presence of unmethylated alleles (Fig. 4C). Indeed, these results are highly correlated with P-cadherin expression because normal breast epithelial cells are negative for this protein. This evidence supported the hypothesis that the transcriptional inactivation of P-cadherin in such cells could be regulated by *CDH3* promoter methylation.

Invasive breast carcinomas. P-cadherin promoter methylation was analyzed in 64 cases of invasive carcinomas (Table 2). Methylation of P-cadherin gene was found in 58% (37 of 64) of invasive carcinomas, whereas P-cadherin unmethylation was found in 42% (27 of 64; Fig. 4D; Table 2). When these results

were correlated with P-cadherin expression, a statistically significant association was found between these variables: 71% of P-cadherin-negative cases were methylated, whereas 65% of positive cases were unmethylated ($P = 0.005$; Fig. 4E; Table 2). Interestingly, six out of seven cases showing partial methylation (with both methylated and unmethylated alleles) were negative for P-cadherin expression, reinforcing the correlation that was observed. Thus, the transcriptional control of P-cadherin in human breast cancer might be associated with changes of *CDH3* CpG island promoter methylation.

Discussion

Over the last 5 years, research on breast cancer has suggested the use of many new prognostic markers. Some of these markers could raise questions, not only about divergent phenotypes, but also about different histogenesis. One of these examples is P-cadherin, which has been identified as a possible valuable indicator of poor prognosis in breast cancer patients (12). In this study, confirming the results obtained by others (11, 12, 29, 34), P-cadherin was indeed significantly expressed in high-grade, ER-α-negative, and highly proliferative invasive breast tumors. Furthermore, a shorter patient disease-free and overall survival in P-cadherin-positive invasive carcinomas was found using the statistical univariate analysis done.

The phenotype acquired by this specific subset of tumors is actually classified as myoepithelial/basal-like, which means the acquisition of molecules frequently restricted to the myoepithelia of normal breast tissue and loss of the ones expressed by epithelial cells (35). These characteristics have indeed been already associated with poor patient survival (35), confirming that P-cadherin is one of the proteins related to biological aggressiveness in breast carcinomas. In a recent report from our group regarding the involvement of P-cadherin in cell invasion, we have shown that this molecule has an effective proinvasive activity in the MCF-7/AZ breast cancer cell line, through its interaction with signaling proteins bound to the juxtamembrane domain (13). In a way, these results give an explanation as to why P-cadherin is correlated with poor prognosis in breast cancer patients. Accordingly, Taniuchi et al. also showed that overexpressed P-cadherin/*CDH3* promotes the motility of pancreatic cancer cells by its interaction with p120ctn and consequent activation of Rho-family GTPases (36). Based on these results, the blocking of P-cadherin activity, or its associated signaling, could probably be a novel therapeutic approach for treatment of aggressive breast carcinomas.

We have already found a direct link between P-cadherin expression and the lack of ER-α signaling in breast cancer cells, showing that P-cadherin expression depends on an estrogen-independent cell environment (13). Additionally, genetic or epigenetic alterations in the P-cadherin gene is likely to regulate the behavior of neoplastic cells as compared with that of normal epithelial cells: cell-cell interactions may be changed, leading to modified intercellular communication and, consequently, altered intracellular signaling (37).

DNA methylation of promoter CpG islands has been recognized as an important mechanism for regulation of gene expression and transcriptional modification in mammals. Although considerable work has been done on the epigenetic control of tumor suppressor genes, little is known about the potential role of promoter CpG demethylation in the activation of oncogenes (32).

The present study showed a statistically significant correlation between the methylation patterns of the *CDH3* promoter region of P-cadherin and its aberrant protein expression levels in breast carcinomas. This methylation status of the DNA chromatin in P-cadherin promoter region might play a role in the ability of transcription factors to bind to the transcription start site, thus regulating mRNA transcription, and consequently its protein expression. Our results are supported by Jarrard and collaborators' data in prostate cancer cell lines (14). They showed that *CDH3* promoter methylation occurs in P-cadherin-negative cell lines, but not in cell lines expressing this gene, and showed a complete ablation of P-cadherin transcriptional activity when methylation was present to any extent within the GC-rich promoter region. This was also described in a recent study done in melanoma cell lines (28), and is in keeping with reports on the role of promoter methylation for transcriptional regulation of tissue-specific genes (38). Moreover, the treatment of an extensive culture of MCF-7/AZ breast cancer cell line with 5-Aza-2′ -deoxycytidine demethylating agent induced the expression levels of P-cadherin mRNA and protein. These results provided clear evidence that the observed correlation *in vivo* is backed up by experimental *in vitro* data.

In contrast with our results, these same authors have reported the absence of a correlation between *in vivo* P-cadherin expression in prostate adenocarcinomas and the methylation status of the *CDH3* promoter (14). In prostate tissue, P-cadherin is strongly expressed on basal epithelial cells and is completely negative in luminal epithelial cells. However, in contrast with breast cancer, this protein was absent in the prostate cancers analyzed in this study. Additionally, the methylation status of a P-cadherin CpG island was examined in 12 normal and matched primary prostate cancers, using methylation-sensitive enzymes. Although all the restriction sites analyzed were found to be unmethylated in P-cadherin-positive normal tissues, no detectable methylation was shown in any tumor sample, showing no correlation with the absence of P-cadherin expression (14). In our opinion, the conflicting results obtained by these authors can be explained by two reasons: (*a*) the number of cases that were studied and (*b*) the distinct methodology that was used. Also in our series, we obtained cases that did not show correlation between the promoter methylation status and the protein expression: 29% of the P-cadherin-negative cases showed unmethylation pattern, and 35% of the positive cases were methylated. However, using statistical analysis in a large tumor series, we were able to find a significant correlation between P-cadherin expression and gene hypomethylation. Statistics using only 12 cases cannot be representative of the methylation process as a regulator mechanism occurring in cancer cells. Besides, these authors claim that the normal prostate tissue is completely unmethylated for the P-cadherin gene in all the restriction sites analyzed. Because normal tissue includes basal P-cadherin-positive cells as well as luminal P-cadherin-negative cells, it was expected to find both methylated and unmethylated alleles in these samples. Therefore, we used methylation-specific PCR assays in order to analyze larger DNA fragments, and also microdissected our samples to get more homogenous cell populations.

Furthermore, supporting our hypothesis, the laser-microdissected normal breast epithelia showed complete methylation

of the *CDH3* promoter region in the five cases studied, suggesting that P-cadherin protein expression is repressed in normal epithelial cells by this specific molecular mechanism. Based on these results, we can suggest that, during breast carcinogenesis, progressive hypomethylation of *CDH3* alleles occurs, which induces its expression in some mammary carcinomas, where its functional activity induces cancer cell invasion and motility (13, 36). This progressive *CDH3* promoter hypomethylation is clearly seen in cases presenting partial methylation, which were still negative for P-cadherin expression like the normal epithelial cells.

Cancer-associated DNA hypomethylation is as prevalent as cancer-linked hypermethylation, although its biological significance in carcinogenesis is less understood. There are several examples of other tumor-overexpressed genes, which become promoter-hypomethylated during carcinogenesis, supporting the data observed within *CDH3*. The cyclin D2 gene is overexpressed in a subset of gastric carcinomas, and Oshimo et al. have shown that DNA hypomethylation is a mechanism underlying the increased expression of cyclin D2 in cancer cells and that its progressive demethylation may be involved in the development and progression of gastric carcinoma (32). Also, the melanoma antigen (*MAGE*)–encoding genes are expressed in various tumor types via demethylation of their promoter CpG islands (39), which are silent in all nonneoplastic tissues except for the testis and placenta. Honda et al. have found that *MAGE-A1* and *-A3* demethylation occurs during progressive stages of gastric cancer, and tended to be associated with a worse patient prognosis (40). Also in breast cancer, urokinase-type plasminogen activator, which is only expressed by highly invasive cancer cells and has been implicated in tumor motility, invasion, and angiogenesis, was also found to be the result of hypomethylation of its codifying gene (41–43).

In conclusion, although there is just a small number of genes that have been shown to be transcriptionally activated by DNA demethylation, in comparison with the list of tumor suppressor genes that are silenced by hypermethylation in cancer cells, all these studies are consistent with the hypothesis that hypomethylation of critical genes plays a role in cancer invasion and metastasis. Based on this, the methylation inhibition of these genes, like urokinase-type plasminogen activator and P-cadherin in breast cancer, can be used as a novel therapeutic approach to silence their expression and, consequently, to block tumor progression into the aggressive and metastatic stages of the disease.

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